STANDARDIZATION OF THE PRECIPITIN TECHNIQUE AND ITS APPLICATION TO STUDIES OF RELATIONSHIPS IN MAMMALS, BIRDS AND REPTILES

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Serologic techniques are now used in studies of relationships among bacteria, plants (see review by Chester, 1937) and animals. The types of tests commonly employed are the complement fixation, the agglutination and the precipitation reactions. The particular type of study governs the test employed and there are advantages to each one.

Although it is claimed that the precipitin reaction is not as sensitive as other serologic methods, its simplicity and accuracy make it advantageous in studies of animal relationships where large numbers of tests are necessary. The inconsistency of results, as shown in serologic literature, is possibly not due to the unreliability of the precipitin reaction but rather to the differing techniques of the workers who make the tests. While the techniques that were first employed in the precipitin reaction were important and gave interesting results, they have been much improved in recent years and need still further standardization. There should be a uniform method of measuring the end-point (titer), the chemical and physical factors should be constant and the methods of injection should be standardized.

The precipitin reaction is the one that has been most commonly used in the study of animal kinship, though agglutinins, responsible for blood groups, have been employed in the case of apes and man. Comparatively little research has been done on the serological relationships of reptiles or of birds. Nuttall (1904) stated that there was "a similarity of the blood constitution of all birds." He found differences in degree of reactions but these were so slight that a study like that made with mammalian bloods was not possible. Sasaki (1928) claims that he was able to distinguish the Japanese duck (*Anas domestica erecta*) from the Chinese goose (*Anser cygnoides*) with untreated sera and could distinguish the Japanese duck from the Muscovy duck (*Cairina moschata*) by specialized sera. These results would be more reliable were the possible error of the tests determined. Graham-Smith (1904—see Nuttall, Section VIII) found a closeness of relationship between the Crocodilia and

Chelonia and between the Ophidia and Lacertidia. The reactions indicated that the former groups stood more closely related to the Aves.

Ehrhardt (1929) reviewed the whole field very completely. Some important research has been done recently showing the significance of the tests in determining uncertain relationships. Boyden and Noble (1933) applied the reaction to a study of relationship among several amphibians and clarified the status of these forms. Eisenbrandt's (1938) extension of these methods to chiefly parasitic helminthes is of value in showing that lower animals with only relatively small amounts of circulating proteins may also be successfully studied.

Zuckerman and Sudermann (1935) reported on "Serum relationships within the family Cercopithecidae." As part of their conclusions they state, ". . . their findings support the view . . . that the serum-precipitin test is of limited value in tracing phylogenetic relationships." Their conclusions from their data are not justifiable, however, since these data were obtained by a technique which is questionable.

Magnus (1908) noted that antisera specificity was lessened by continued injections of an antigen. Wolfe (1935, 1936) and Wolfe and Baier (1938) emphasized the importance of the effects of injection methods on the specificity and aspecificity of the serum precipitin tests. It was shown that the type of response is greatly influenced by the method of injection, the specificity being enhanced by limiting the amount of protein injected. With this technique they found greater distinctions among more closely related forms and less "extra-group" reactions. These results suggested that even greater specificity might be possible if the amount of protein injected was reduced to a minimum. It is known that exceedingly small quantities of protein may cause the formation of precipitins in rabbits. Hektoen and Cole (1932) were able to produce antisera of fairly high titers in rabbits by injecting as low as .00029 gram of an ovalbumin solution.

The purpose of this research is: (1) to determine whether precipitin specificity can be increased by limiting the quantity of protein injected into rabbits to a minimum necessary for antibody production; (2) to discover whether a serological relationship study on birds and reptiles is practicable; (3) to attempt to further standardize precipitin technique.

Methods and Materials

Healthy adult rabbits of mixed breeds and weighing approximately three kilograms were injected intravenously with serum proteins of ten species of mammals, five species of birds and one species of reptiles. Listed in Table I are the scientific and common names of the animals

TABLE I

Antigens used in experiments

Scientific Name	Common Name	Grams protein per 100 cc. serum
Homo sapiens *	Man	6.843
Pans satyrus	Chimpanzee	6.677
Macacus rhesus	Rhesus macaque	5.425
Macacus sinicus	Bonnet macaque	6.926
Papio hamadryas	Hamadryas baboon	5.530
Lasiopyga mona	Mona guenon	8.616
Cebus capunicus	Capuchin monkey	7.198
Bos taurus *	Ox	6.825
Bison bison *	American buffalo	7.982
Capra hircus *	Domestic goat	6.593
Ovies aries	Domestic sheep	6.051
Odocoileus virginianus	White-tailed deer	5.843
Tamiasciurus hudsonicus *	Red squirrel	6.409
Sciurus carolinensis *	Gray squirrel	8.750
Sciurus niger	Fox squirrel	6.560
Citellus tridecemlineatus	13-lined ground squirrel	5.096
Tamias striatus	Eastern chipmunk	5.139
Marmota monax	Ground hog	6.260
Rattus norvegicus	White rat	7.052
Felis domesticus	Cat	6.825
Canis familiaris	Dog	6.364
Gallus domesticus *	Chicken	4.676
Columba livia *	Pigeon	3.133
Strix varia	Barred owl	4.070
Larus argentatus	Herring gull	3.070
Ardea herodias	Great blue heron	3.550
Mergus merganser	American merganser	3.800
Anser anser	Domestic goose	4.387
Anas boschas	Domestic duck	4.676
Alligator mississippensis	Alligator	5.158
Phrynosoma cornutum	Texas horned toad	4.389
Chelydra serpentina *	Snapping turtle	3.654
Chrysemys picta	Painted turtle	3.446
Emys blandingii	Blanding turtle	2.299
Clemys insculpta	Wood turtle	3.550
Thamnophis sertalis	Common garter snake	5.405

* Used for production of antisera and test antigens, others only for test antigens.

from which the blood proteins were secured. The amount of protein per 100 cc. was determined on the whole serum by the Folin-Wright (1919) modified macro-Kjeldahl method. Corrections were not made for non-protein nitrogen. Table II records antisera production data for some of these species. The quantity of protein injected and titers are based on protein calculated from total nitrogen.

The amount of protein injected per animal in the first series has been as low as .000786 gram. This is an exceedingly small amount and not always do such quantities injected into rabbits yield potent antisera. When a second series of injections was given the dosage was usually about one-half the initial series dosage. At present a standardized injection technique is being followed. Rabbits are injected with .001 gram

TABLE II

Antisera production data

Rabbit No.	Serum protein injected	Injected dil. of solution	Total protein injected	Bled—days after last injection	Titer (dil.)
		cc.	*grams*		
205	Red squirrel	6.0 (2%)	.00768	12	512,000
215		1.2 (2%)	.001536	12	512,000
136		3.2 (1 : 1)	.102544	14	51,200*
217	Gray squirrel	1.0 (1 : 1000)	.001	10	0
		1.0 (1 : 1000)	.001	12	16,000
		0.75 (1 : 1000)	.0075	8	512,000
223		2.0 (1 : 1000)	.002	10	128,000
125		2.25 (undil.)	.19687	10	51,200*
A11	Ox	1.5 (1 : 500)	.003	2	0
				4	0
				6	16,000
				9	1,024,000
A 9	Buffalo	1 mg./kil.	.0034	10	256,000
A10	Goat	1 mg./kil.	.0034	10	128,000
185	Human	5.75 (2%)	.00786	12	51,200*
77		3.0 (undil.)	.20531	12	102,400*
209		0.6 (2%)	.00082	13	128,000
221		2.0 (1 : 1000)	.002	10	512,000
225	Chicken	2.0 (1 : 1000)	.002	12	0
		0.6 (1 : 500)	.0012	7	512,000
228		0.75 (1 : 1000)	.0015	8	1,024,000
229	Pigeon	1.0 (1 : 500)	.002	10	512,000
222	Snapping turtle	1.0 (1 : 500)	.002	10	0
		0.6 (1 : 500)	.0012	7	256,000

* This notation indicates a serial dilution from a 2 per cent standard—all the rest are from standard solutions containing .001 or .002 gram per cc.

of protein per kilogram body weight distributed over three injections. The injections are made on alternate days. When additional series are necessary the amount administered is one-half that of the initial series given in two injections on alternate days.

Ring tests were used exclusively in determination of the titer. The test consisted of having a duplicate series of serological tubes containing

0.5 cc. of antigen in serial dilution with 0.1 cc. of antiserum layered beneath the antigen. The titer was determined after incubation in a water bath at a temperature of 37.5° ± 1° C. The possible error in reading the reactions was ± 1 tube. Several experiments consisted of readings at intervals during the sixty minutes in order to study the progression of the titers. Unless definitely specified, the antisera were used undiluted or 1.0 cc. was diluted with 0.25 cc. of a buffered physiological saline solution. This small dilution did not have any effect on the strength of titers in the experiments.

In the earlier experiments the serial dilutions were made from standard test antigens which were 2 per cent solutions. The latter were made by diluting 1.0 cc. of the undiluted blood with 49.0 cc. of buffered saline giving a 1 : 50 dilution. Since the protein content of the antigens varied

TABLE III

Anti-human sera

Antigen serum proteins of	77	185*	209	221
Man	102,400	51,200	128,000	512,000
Chimpanzee	25,600	12,800	128,000	256,000
Rhesus macaque	12,800	1,200	0	64,000
Bonnet macaque	6,400	1,600	0	64,000
Baboon	25,600	400	0	256,000
Mona guenon	12,800	400		128,000
Capuchin monkey				16,000
Rodentia		0		0
Artiodactyla	0	0	0	4,000
Carnivora	0	0		1,000

* Titers from a 2 per cent solution of undiluted antigen.

from 4 per cent to 9 per cent the titers need adjustment. In the later experiments all serial dilutions are made from standard solutions that contain either .002 or .001 gram protein per cc. thus making all titers comparable.

EXPERIMENTAL DATA

Effects of Amount of Antigen Injected on the Specificity of the Antisera Produced

One of the main purposes of this paper is to show the variance in the degree of the homologous and heterologous precipitin reactions when one factor, the injection procedure, is varied. Antisera 77, 185, 209, 221 are anti-human sera whose reactions are recorded in Table III. Serum No. 77 was produced by injection of .21594 gram protein while the rabbit producing No. 209 received only .00082 gram of protein. (Reference

should be made to Table II for exact detail on quantity of material injected in the experiments to be described.) Of the four antisera 209 is by far the most specific, giving reactions only with chimpanzee and human blood antigens. No. 185 is more specific than 221 even though the latter received one-third the amount of protein given the former.

Injections of the more minute quantities (similar to 185 and 209) usually gave reactions illustrated by these two and more rarely reactions like 221. On the other hand, when 3 cc. of undiluted serum was injected, the most usual response was like that given by No. 77.

Sera 77 and 221 yielded primate group reactions but No. 185 resulted in a sub-grouping showing that the blood proteins of man and chimpanzee are more nearly related to each other than to the blood of the monkeys. Serum 209 was an extremely specific one, giving reactions with only chimpanzee and the homologous antigens. Such antisera would be of great value to the medico-legal workers, as well as to the student dealing with man's closest relatives.

The reactions of three anti-red squirrel and three anti-gray squirrel sera are shown in Table IV. Serum 136 gave a Sciuridae group reac-

TABLE IV

Anti-squirrel sera

Antigen serum proteins of	Anti-red squirrel			Anti-gray squirrel		
	136*	205	215	125*	217	223
Red squirrel	51,200	512,000	512,000	2,400	64,000	0
Gray squirrel	25,600	0	128,000	51,200	512,000	128,000
Fox squirrel	51,200	0	128,000	51,200	256,000	2,000
13-lined gray squirrel	12,800	0	0	2,400	4,000	0
Chipmunk	51,200	0	0	6,400	8,000	0
Ground hog	51,200	0	0	3,200		0
Rat	0	0		0	0	0
Artiodactyla	400		0	200	4,000	0
Primata	400			400	4,000	0

* Titers from a 2 per cent solution of undiluted antigen.

tion with no sub-grouping evident, but No. 125, an anti-gray squirrel serum, reacted much more weakly with the blood of red squirrel and other sciurids but could not be differentiated from fox squirrel blood. Both these sera were produced by injection of a much larger quantity of protein than were the other four listed in the table. The latter all show that a definite difference exists between red squirrel blood and that of the fox or gray squirrel bloods. Sera 215 and 217 subdivide the Sciuridae reactions into three sub-groups. Serum 205 is very specific and gave no cross reactions at all and No. 223 allowed for distinction between the

TABLE V

Anti-aves sera *

Antigen serum protein of	Anti-chicken		Anti-pigeon
	225	228	229
Chicken	512,000	1,024,000	0
Pigeon	4,000	256,000	512,000
Goose	16,000	256,000	0
Duck	8,000	256,000	0
Merganser	0		
Great blue heron	8,000	0	0
Barred owl	8,000		
Herring gull	0		0
Snapping turtle	2,000		0
Painted turtle	0	0	0
Alligator	0	0	
Common garter snake	0	0	0
Cryst. egg albumen of chicken	0	0	0

* Fowl and reptile data are from a thesis by Miss Bernice Cohen done under the supervision of the author and used with her permission.

two closely related forms of gray and fox squirrels. This latter result is very rarely secured by the precipitin test with native antisera.

The variation in results that were secured with different dosages do not subtract from the value of the precipitin test but, on the other hand, give it greater possibilities. The differences in reactions seem to imply that a distinction can be made among genera within a family and possibly

TABLE VI

Anti-snapping turtle

Antigen-serum Protein of	*No. 222*
Snapping turtle	256,000
Painted turtle	64,000
Blanding turtle	16,000
Wood turtle	16,000
Alligator	0
Horned toad	0
Common garter snake	0
Aves (7 species)	0
Cryst. egg albumen of chicken	0

species within a genus as well as among larger groups. Similar results have been reported previously but there now seems to be a method whereby these results can be secured consistently. It would be advisable to have many antisera for each group studied in order to insure significant results.

It might be stated that it is believed that the variability of results is not attributable to the innate characteristics of the test but is probably

TABLE VII

Effects of Length of Incubation Period and of Dilution of Antisera

		Serum Undiluted				Dilution	
						1 : 3	1 : 5
Incubation Period—Minutes	1	5	10	30	60	60	60
		Anti-buffalo—A9					
Buffalo	0	64,000	128,000	128,000	256,000	512,000	128,000
Ox	0	64,000	128,000	128,000	256,000	512,000	128,000
Sheep	0	0	0	4,000*	64,000	4,000	1,000*
Goat	0	0	0	4,000	128,000	4,000	2,000*
Deer	0	0	0	32,000	128,000	4,000	
		Anti-goat—A10					
Goat	0	0	0	64,000	128,000	0	
Sheep	0	0	0	64,000	64,000	1,000	
Buffalo	0	0	0	0	0		
Ox	0	0	0	0	0		
Deer					0		
		Anti-beef—A11					
Beef	64,000	128,000	512,000	1,024,000	1,024,000	512,000	512,000
Buffalo	64,000	128,000	512,000	1,024,000	1,024,000	256,000	256,000
Sheep	0	0	0	16,000	128,000	0	
Goat					128,000	0	
Deer	0	0	0	64,000	256,000	0	

* Readings not clear.

due to the antibody producer, in this case, the rabbit. It would be an ideal condition were one able to secure a group of rabbits with similar potentialities for antibody production.

Tests with Avian and Reptilian Blood Sera

There is a meagerness of serological data with respect to reptilian and avain bloods probably due to the conception that the animals within each of these groups are very closely related to each other and are not suitable for study by means of the precipitin reaction. The data listed in Tables V and VI contradict this contention. There are indications that the reactions of anti-avian and anti-reptilian sera produced by using very small amounts of proteins may be useful in confirming present morphological data and thus aiding in the clarification of disputed relationships.

Effects of Incubation Period and Dilution of Antisera on Titers

Previous workers have considered such factors as the length of incubation period and the effect of dilution of the antisera on the homologous and heterologous reactions. The usual method is to make readings after 1 hour incubation at a temperature of 37° ± 1° C. when the ring test is used and the antisera are used undiluted or diluted to various degrees.

Table VII shows the reactions of three anti-Artiodactyla sera which illustrate both time and dilution effects. These antisera readings at 5, 10, and 30 minutes usually showed much greater specificity than did the readings at 60 minutes. Serum A 9 lost its distinguishing characteristic at 60 minutes so that the reactions of buffalo and ox could not be distinguished from deer and goat. It should be noted that antisera A 10 and A 11 show that beef or buffalo can be markedly differentiated from either goat or sheep bloods even at 1-hour readings. Heretofore these great differences have not been shown (with rare exceptions) when undiluted and untreated antisera against the serum proteins were used. By using very small quantities of blood for injections, results similar to, or showing greater differentiation, are consistently secured.

The dilution of antisera with physiological saline is first noted to have an effect on the antigens more distantly related (according to taxonomical schemes). These antisera became much more specific by diluting them as low as 1:3 (2 parts saline and 1 part antiserum). With beef antiserum A11 the reactions of sheep, goat and deer antigens disappeared at antiserum dilutions of 1:3 while the buffalo antigen titer remained similar to the homologous reaction. Such low dilutions show these effects only when the antisera have been produced by the methods described; that is, injections of minute quantities of proteins.

Discussion

Data are presented that show how a greater uniformity of results may be obtained with the serum precipitin reaction. These data further supplement previous work of the author in regard to the specificity and aspecificity of the test. The consistency of the specificity of the reaction with the whole serum proteins of mammalian, avian and reptilian bloods is greater than heretofore reported with untreated rabbit antisera. A distinction of the bloods of such closely related forms as ox and sheep, red squirrel and gray squirrel, several species of turtles and of a number of birds was possible.

The cause of this greater specificity is believed to be the small quantity of protein injected. A standardized quantity is now used; the amount injected in the first series is .001 gram of protein (based on total nitrogen) per kilogram body weight and distributed in three injections. If the titer of the antiserum resulting is insufficient the animal is reinjected with .0005 gram per kilogram body weight given in two injections. This amount injected is thought to be approximately the smallest amount of the antigen used that will regularly cause antibody formation.

The results are not always similar and therefore it seems necessary to produce a number of antisera against a particular antigen in studying relationships and choose those which have the desired specificity. With the present procedures employed the antisera against a particular kind of antigen fall into groups. The reactions of the different groups differ quantitatively but not necessarily qualitatively. Not only are relationships shown but the degree of such relationships can be studied with greater accuracy than before. Morphology does not allow for such a type of approach to the classification of animals. These differences can best be understood by referring to the tables on rodent or primate reactions.

The high specificity secured with a few of the antisera even when used untreated should interest the worker concerned with the medico-legal aspects. Antisera that give reactions only with very closely related bloods should be of much value and no doubt the evidence so secured would be readily accepted by our courts. Such antisera are comparatively easy to produce if the amount of protein injected is reduced to the approximate minimum.

The titer of the reaction at 1 hour incubation did not show the marked differences that were shown after incubating for shorter periods. This factor, the length of incubation period, has not been used with any uniformity for distinguishing closely related proteins. When antisera are produced by the injection of very minute quantities of protein the homologous blood and very closely related ones give ring tests with high titers after 1, 5, 10 or 20 minutes incubation and after that period the blood of more distantly related forms becomes positive. These reactions, when properly performed, are usually very distinct and consistent.

Attempts were made to use readings after incubation of more than 1 hour. These readings are not so clear-cut and not consistent and so are considered unreliable.

The second *in vitro* factor which enhanced the specificity of the reaction, that is, dilution of the antisera, showed a direct correlation with

the results secured by studying the period of incubation. Boor and Hektoen (1930) and Hektoen and Boor (1931), using antihemoglobin sera, found that dilutions with 3 parts of saline or normal rabbit serum eliminated extra specific reactions. Satoh (1933) demonstrated that dilution of 1 : 10 or less affects the titer of an antiserum when it is produced by few injections with a small quantity of material, and dilution of 1 : 25 or more affects the reactions when the antisera result from injection of larger quantities given in a greater number of injections. In Satoh's report the dilution seemed to have a similar effect upon the heterologous and homologous reactions. Wolfe (1933) reported that dilutions of 1 : 2 make the readings of the " ring " clearer but that dilutions of 1 : 10 do not always eliminate extra-group reactions. These antisera were produced by injection of a relatively large quantity of antigen.

The antisera that were used in the present work were easily affected by small dilutions with buffered saline. The lowest dilution to have an effect was a 1 to 1 (1 part saline) dilution but with some antisera it was necessary to dilute with 2 or 3 parts of saline in order to show decrease in reaction. The dilution always lowered or eliminated the heterologous titer first and one could thus verify the dissimilarities between such closely related bloods as ox and sheep, or man and monkey. But even a higher dilution could not distinguish consistently between ox and buffalo or man and chimpanzee for the dilutions had similar effects upon both titers.

The data presented in this paper are fairly consistent. The methods reported are easily followed and it seems they insure a uniformity of results. The different approaches used resulted in a partial solution of the primary problem concerned; namely, the degree of similarity of the blood proteins of some of the more closely related mammals, birds and reptiles. It seems very possible that a reliable serological relationship study of birds and of reptiles can be accomplished. Additional data may aid in clarifying the " taxonomic confusion " of birds.

Finally—a plea is made to the workers using the serologic method, especially the precipitin technique. It is desirable to obtain uniform and reliable results and one should use the methods that are best adapted to the particular problem. The earlier workers' contribution was and is invaluable but not necessarily the only one that is of use. A modification of the earlier methods is in order and only when one uses the best possible method for the particular study will the results be consistent and therefore biologically significant.

Summary

1. Antibodies were produced in rabbits by injecting very small quantities of serum proteins of mammals, birds and reptiles.

2. Antisera produced by injection of a minimum quantity of antigen were the most specific as determined by the "ring" method.

3. Occasionally the serum proteins of such closely related animals as ox and sheep or gray squirrel and red squirrel could be distinguished.

4. Antiserum dilutions as low as 1 : 3 at times eliminated the heterologous reaction.

5. The "ring" appeared in the homologous and very closely related protein solutions earlier than in the more distantly related ones when incubated at temperatures of 37° ± 1° C.

6. It was not possible to distinguish between the buffalo and ox serum proteins or between the goat and sheep serum proteins.

7. Serological relationship studies of birds and reptiles seem feasible.

8. The procedures have not been entirely new but the necessity for uniformity and standardization of methods has been emphasized.

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